The Response

1. **Drawings**

Applicants are deferring the submission of Formal Drawings until the Examiner allows the application.

2. **Sequence Rules**

Applicants have submitted paper and computer readable format of sequence listing on August 8, 2000.

3. **Objection to the Specification**

The specification is objected to because of informalities. The objection is overcome in parts in view of the amendments and in parts traversed. Applicants have amended the specification to insert the priority claim in the first paragraph of the specification and the generic name of TWEEN 20®.

Applicants do not agree with the Examiner that the incorporation of references at pages 3 and 4 of the substitute specification is improper for the following reasons. A copy of pages 191-210 of "Human Chromosomes" which is cited in the application is submitted herewith. The reference discloses that Comparative Genomic Hybridization (CGH) is a conventional method known since 1990 by which a comprehensive analysis of imbalanced chromosomal material of entire genomes is permitted (Boyle *et al.*, 1990; Kievits *et al.*, 1990). The comparative genomic hybridization (CGH) methods were well known to those skilled in the art at the time the present application was filed. Therefore, the incorporation of references for the CGH method is proper and the Examiner's objection should be withdrawn.

4. **Rejections under 35 U.S.C. § 112, First Paragraph.**

Claims 1-8 are rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Examiner states that pages 2-3 of the specification disclose that the identification of any change in copy number of a target sequence is achieved via performing comparative genomic hybridization; the specification does not enable this form of detection as essential subject matter has been improperly incorporated by reference. The Examiner's rejection is respectfully traversed.

As discussed above, the comparative genomic hybridization (CGH) methods were well known to those skilled in the art at the time the present application was filed. A specification need not disclose what is well known in the art. (*Hybritech, Inc., v. Monoclonal*

Antibodies, Inc., 231 USPQ 81, 94 (Fed. Cir. 1986) An omission of minor details does not cause a specification to fail to meet the enablement requirement.

The CGH method is disclosed in *Human Chromosomes*. In the CGH method, a genomic DNA to be analyzed (test DNA) is labeled and cohybridized with differentially labeled normal DNA (control DNA) under suppression hybridization conditions to metaphase chromosome spreads from normal cells.

In the Example of the present application, a normal genomic DNA is tag-labeled by DOP PCR (step 1) and modified by digoxigenin-11-dUTP (step 2); the product obtained is control DNA. The control DNA is *in situ* hybridized on interphase nuclei of line Colo 320 (step 3). Then the hybridized nuclei are isolated and subjected to DOP PCR (step 4); the product obtained is test DNA. The test DNA is cohybridized with the control DNA under suppression hybridization conditions to metaphase chromosome spreads from normal cells (step 4: CGH analysis). Applicants have provided a working example, which includes working conditions for the claimed invention. The CGH method is a well-known method at the time of filing this application; thus it is not necessary to describe the details of the CGH method because Applicants have incorporated the references that describe the CGH method into the application.

In the working example of the application, Applicants describe the reagents, buffers and solutions for preparing a tag-labeled sequence. Applicants also describe the denaturation conditions of the genomic DNA of the Colo320 interphase nuclei, the hybridization conditions for the *in situ* hybridization, and the washing and detection conditions of the hybridized cell nuclei. Further, Applicants describe the conditions for isolating the hybridized nuclei. The CGH analysis is described in the cited references that are incorporated by references. Following the example taught in this application, an ordinary skilled person would be able to practice the invention. Although the Examiner asserts that the claimed invention is sensitive to the conditions employed in a hybridization reactions, the optimization of hybridization reactions is routine to a skilled artisan and does not constitute an undue experimentation.

Therefore, the §112, first paragraph rejection of Claims 1-8 should be withdrawn.

5. Rejections under 35 U.S.C. §112, Second Paragraph.

The Examiner has rejected claims 1-8 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to point out and distinctly claim the subject matter which application regards as the invention. The rejection is overcome in parts in view of the amendments and traversed in pares. Claim 1 is amended to delete "numerical" and Claim 4 is amended to delete "small." As to "tag", the rejection is traversed. The expression "tag" refers to the fact that the primers can be degenerative (universal) primers, *i.e.*, primers which can bind to many different sites of a cell DNA. Examples of such primers are DOP or SiA primers (see specification at page 2, liens 9-11). Therefore, the §112, second paragraph rejection of Claims 1-8 should be withdrawn.

Claims 1-7 are rejected under 35 U.S.C. §112, second paragraph, as being incomplete for omitting essential steps. Claim 1 is amended to include the step of cohybridizing the DNAs from (a) and (c) to metaphase chromosome spreads from normal cells under suppression hybridization conditions. Therefore, the amended Claims 1-7 do not omit any essential step.

The Examiner also states that claim 8 is confusing with respect to just how the primers relate to the amplified DNA. Claim 8 is amended to recite that a kit comprising DNAs flanked by tag primers that are amplified from cells that have no known changes in their DNAs. Therefore, the §112, second paragraph rejection of Claim 8 should be withdrawn.

In view of the above amendments, Applicant respectfully requests the withdrawal of rejections under 35 U.S.C. §112, second paragraph.

CONCLUSION

It is now believed that the claims are in condition for allowance and advancement as such is earnestly requested. Should any questions arise in connection with this submission which may be resolved by a telephonic interview, the Examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

Date: September 25, 2000

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HUMAN CHROMOSOMES

PRINCIPLES AND TECHNIQUES

SECOND EDITION

RAM S. VERMA / ARVIND BABU

1 will require 2 to 3 hours depending on the number of specimens to be hybridized.

3. Washing and detection of probes (day 2) will take 2.5 to 3.5 hours, and longer if amplification is required. Additional time will be needed to examine the slides at the microscope. Examples of the types of results that can be expected using the protocols provided are given in Figs. 6.1 through 6.5 (color plates 4 through 8).

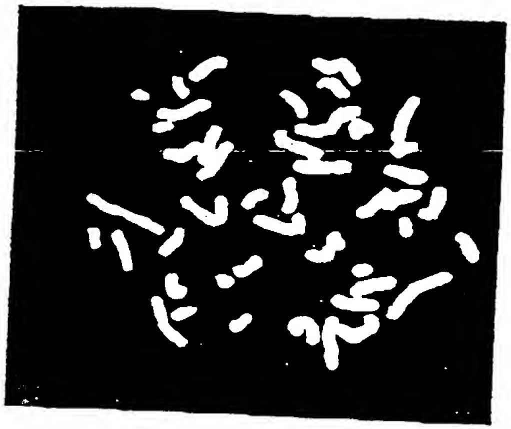


Figure 6.2.
Hybridization of an alphoid DNA probe (p82H) to the centromeric regions of human metaphase chromosomes. The probe, labeled with biotin by nick translation, was detected with FITC-labeled avidin. Chromosomes are counterstained with propidium iodide (color plate 5).

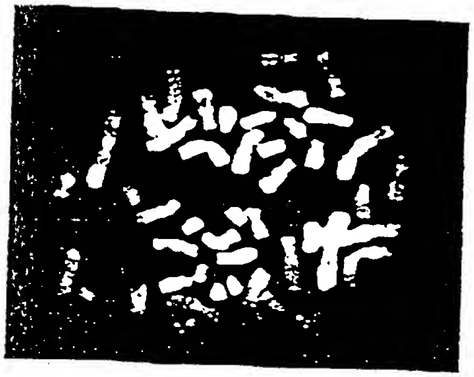


Figure 6.3.
Detection of telomeric sequences on human chromosomes using a biotinylated oligonucleotide probe $(TTAGGG)_n$, generated by PCR (color plate 6). (From Ijdo et al., 1991.)

Figure 6.4.
Mapping of the human microtubule-associated protein (MAP) 1B gene by FISH to 5q13, the chromosomal region encoding the spinal muscular atrophy locus. A phage clone containing the MAP1B gene was labeled with biotin and detected after hybridization with rhodamine-conjugated avidin (red). The chromosomes are R-banded using fluorescein-labeled *Alu* PCR products from human genomic DNA (color plate 7).

COMPARATIVE GENOMIC HYBRIDIZATION

This section was contributed by Peter Lichter, Martin Bentz, Stanislas Du Manoir, and Stefan Joos.

Fluorescence in situ hybridization enables the delineation of targeted chromosomes or chromosomal subregions in a highly specific manner (see Chap. 6). Utilization of this technique for the detection of chromosomal aberrations on metaphase spreads and in interphase nuclei has depended on the application of specific nucleic acid probes. Therefore, the analysis has generally been restricted to a limited number of chromosomal regions. Furthermore, for routine diagnostics, a preknowledge of the regions that might be subject to chromosomal aberrations in certain diseases is mandatory.

In order to allow a more comprehensive analysis of the chromosomal aberrations in a particular cell population, multicolor in situ hybridization protocols are being developed that should permit the detection of an increased number of target regions. A multicolor probe set that permits the differential painting of all 24 human chromosomes is often envisioned. However, the use of such a probe set for a comprehensive analysis of chromosome sets is likely to be limited for several reasons: (1) the painting probes are often not capable of detect-

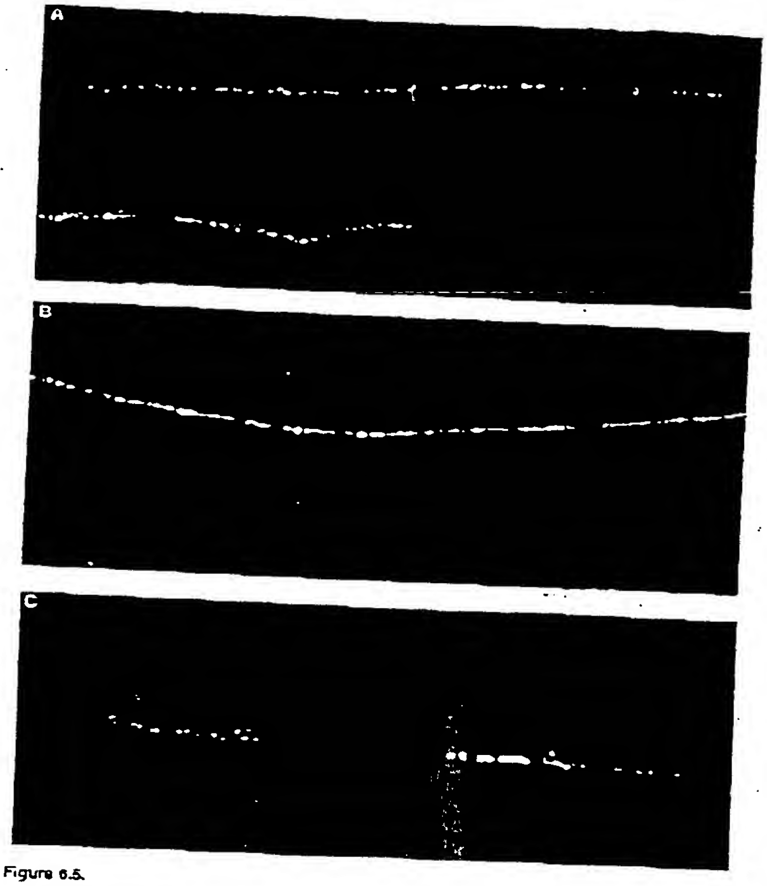


Figure 6.5.

(A, B) Two-color FISH with digoxigenin-labeled α-satellite DNA (red fluorescence) and biotin-labeled CENP-B box sequences (green fluorescence) to highly extended chromatin fibers (ECFs) (Haaf and Ward, 1994b). DNA was counterstained with DAPI. Note the beaded pattern of [illegible] in situ hybridization signals and the regular spacing of CENP-B boxes throughout the linearly extended α-satellite arrays. The micrograph in A shows one α-satellite array along its entire length and part of another array. The micrograph in B shows a hyperextended α-satellite array and, for size comparison, a largely intact interphase nucleus (>30 μm in diameter). In order to visualize the ECF FISH signals, the fluorescence intensity of the interphase nucleus had to be quenched electronically. Therefore, no specific hybridization signals are visible on the nucleus. (C) ECF FISH with three YACs from the 5q34-35 region (Haaf and Ward, 1994a). YACs 786F6 and 935F5, which map to opposite sides of a tumor translocation break point, are labeled with biotin and visualized by green FITC fluorescence. YAC 746B2, which maps to the same side as 786F6, is labeled with digoxigenin and visualized by red rhodamine fluorescence. Note the overlap between 746B2 and 786F6 and the gap between 746B2 and 935F5 (color plate 8).

ing subtle aberrations involving sr. [illegible] chromosomal regions such as small translocated material; (2) metaphase spreads are required from the cells to be analyzed, but in many cases such spreads cannot be obtained and the analysis has to be carried out in the interphase; and (3) the detection of chromosomal aberrations in interphase nuclei depends on the counting and/or spatial relation of hybridization signals, and it is difficult to resolve the signals of 46 painted chromosome domains in a nucleus.

Recently, an alternative approach was introduced that permits a comprehensive analysis of imbalanced chromosomal material of entire genomes (Kallioniemi et al., 1992). This procedure, called comparative genomic hybridization (CGH), is a molecular cytogenetic approach with the potential of detecting chromosomal imbalances in previously unassessable specimens as only DNA is required for the procedure. Therefore, studies analyzing genetic alterations in tumor tissues have been greatly stimulated. Furthermore, it is possible to study even minute amounts of DNA prepared from very few cells by combining CGH with universal polymerase chain reaction (PCR) amplification techniques (Speicher et al., 1993 and Fig. 6.12).

CGH could be considered a brilliant advance in a strategy called reverse painting, in which complete or partial genomes with unknown rearrangements can be analyzed (see Fig. 6.6). The first applications of this approach were presented in studies investigating the chromosomal content of interspecies hybrid cells in more detail (Boyle et al., 1990; Kievits et al., 1990). The whole genomic DNA of a hybrid cell line is used as a probe for suppression hybridization to normal metaphase chromosome spreads of one species. By this procedure, all the chromosomal regions of that species present in the hybrid cell are delineated on the normal chromosome complement, and can be easily identified. Further improvements of this procedure were achieved by using species-specific PCR amplification products from such hybrid lines as probes (Lengauer et al., 1990; Lichter et al., 1990a). Reverse painting is also successfully used to identify the content of marker chromosomes in diseases (Carter et al., 1992). After flow sorting of a marker chromosome, its DNA is used as a probe for in situ hybridization to normal human chromosomes, thus allowing the rapid identification of the origin(s) of the marker chromosome material.

If the whole genomic DNA of cells from one species is hybridized to complete chromosome spreads obtained from normal cells of the same species under suppression hybridization conditions, all of the chromosomes of the metaphase spread are more or less stained. However, when certain chromosomes or chromosomal segments are over- or underrepresented in the probe, stronger or weaker signals, respectively, are expected in the corresponding hybridization target regions (Kallioniemi et al., 1992; Joos et al., 1993; Du Manoir et al., 1993). The possibility of detecting amplified sequences, as well as a loss of chromosomal material, by hybridizing total tumor DNA to normal human chromosomes is illustrated in Fig. 6.7 (color plate 9).

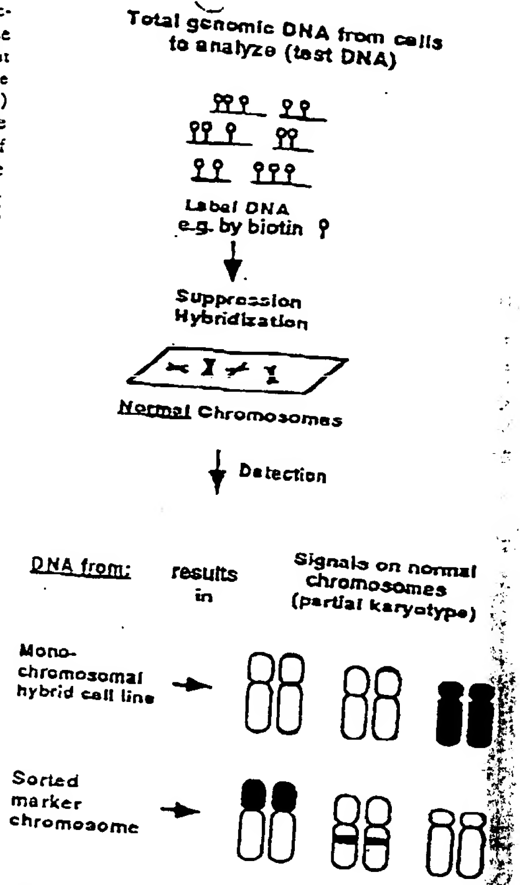

Total genomic DNA from cells to analyze (test DNA)
Label DNA
e.g. by biotin
Suppression Hybridization
Normal Chromosomes
Detection
DNA from:
results in
Signals on normal chromosomes (partial karyotype)
Mono-chromosomal hybrid cell line
Sorted marker chromosome

Figure 6.6.
Schematic illustration of the reverse chromosome painting approach. Genomic DNA from various sources (i.e., a monochromosomal cell line or sorted marker chromosomes) is labeled and hybridized to metaphase chromosome spreads from normal cells. This results in a strong painting of only those chromosomal sequences that are present in the probe DNA (indicated in black).

DNA amplifications recognized in tumor cells by the appearance of minute chromosomes (double minutes or Dmins) or by homogeneously staining regions (HSRs) of aberrant chromosomes (or detected by other techniques such as Southern blot analysis) are readily detected by reverse painting using the genomic DNA of

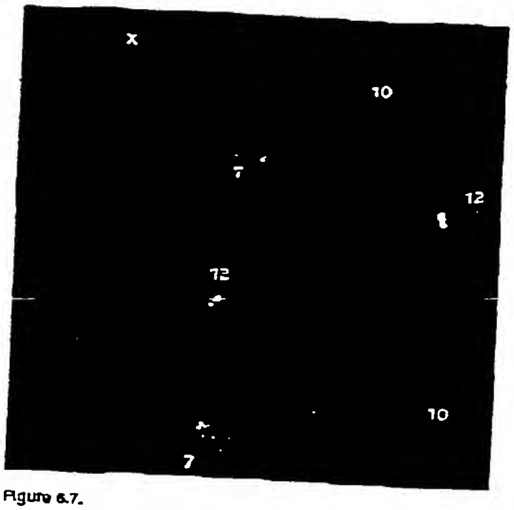


Figure 6.7.
Gray-level CCD image of a metaphase spread after hybridization with DNA derived from a glioblastoma. Amplifications on chromosomes 7p13 and 12 (12q13-q15) are clearly visible. In addition, a weak painting of chromosomes X and 10 indicates an underrepresentation of these chromosomes (color plate 9). (This illustration was taken from Joos et al. [1993] with permission of Springer-Verlag, Heidelberg and New York.)

the corresponding cell population as a probe. The efficiency of this detection was determined by using cell lines with DNA amplifications of known copy numbers and sizes (Joos et al., 1993). Large amplifications, for example, with approximately 24 copies of an amplicon more than 600 kb long, were shown to be visible on some 90 percent of the chromosomal targets (i.e., in the range of the efficiency of in situ hybridization with cosmid probes), whereas the efficiency for smaller amplicons decreased: for example, 20 copies of an amplicon approximately 90 kb long were delineated on approximately 70 percent of the target sequences. However, since the tumor-associated amplifications known to date commonly consist of amplicons more than 100 kb long, this efficacy is clearly sufficient to allow a rapid and comprehensive screening for amplifications.

For a more detailed and accurate analysis of imbalanced chromosomal material within entire genomes, in particular, when there are only subtle deviations from a balanced genome (such as imbalances of small chromosomal regions or chromosomal gains or losses in only smaller proportions of cells), CGH is required. For CGH, the genomic DNA to be analyzed (in the following, termed *test DNA*) is labeled and cohybridized with differentially labeled normal DNA (in the following, called *control DNA*) under suppression hybridization conditions to metaphase chromosome spreads from normal cells (see Fig. 6.8). Test and control DNAs are detected via different fluorochromes. Signal variations are then analyzed by comparing the signal intensity generated by the hybridized test DNA with that from the cohybridized control DNA, rather than by analyzing differences within the test DNA signals along the chromosomes. Thus the control DNA introduces an internal standard, which allows differentiation between variations in signal intensities that are caused by differences in DNA sequence copy numbers from those that are attributable to experimental parameters. For a comprehensive analysis of the chromosomal imbalances, this standardization is very important, since the source of variation of signal intensities along the chromosomes by experimental parameters can be multifold. It can include differential denaturation of chromosomal regions with different base composition (e.g., GC- versus AT-rich regions) or the result of different chromatin packaging, as well as differential hybridization of probe sequences due to base composition and target accessibility.

There is likely to be a second important reason for the need to cohybridize the control DNA. It is generally believed that the probe DNA covers only part of the denatured chromosomal target sequences. Whereas sequences underrepresented in the probe will cover only a portion of this part, overrepresented sequences will result in more extended coverage. In principle, however, if only test DNA is used, long hybridization times (or higher concentrations of DNA in the hybridization solution) will lead to a (almost) complete coverage of all targets, thus not allowing the detection of diagnostic signal variations. The inclusion of differentially labeled control DNA, as performed in CGH, provides probe sequences specific for the same chromosomal targets, and so a constant ratio of test and control DNA is implemented. Since the hybridization of both DNAs follows the rules of stochastics, this ratio must stay constant even if the chromosomal target region is close to saturation with probe sequences. Hence, comparative genomic hybridization seems to be essential for a robust protocol detecting chromosomal imbalances by reverse painting with total genomic DNA.

Test and control DNA probes for CGH can be labeled either directly with fluorochromes by incorporating fluorochrome-coupled nucleotides, or indirectly, that is, with reporter groups such as biotin or digoxigenin detected via fluorochrome-coupled reporter binding molecules. Whereas directly labeled probes result in smooth signals along the chromosomes, which are preferred in principle for assessing the signal intensities along a chromosome, in our hands, indirect labeling/detection procedures yielded highly reproducible results of good quality. Therefore, in the protocols, direct as well as indirect labeling procedures are described. We com-

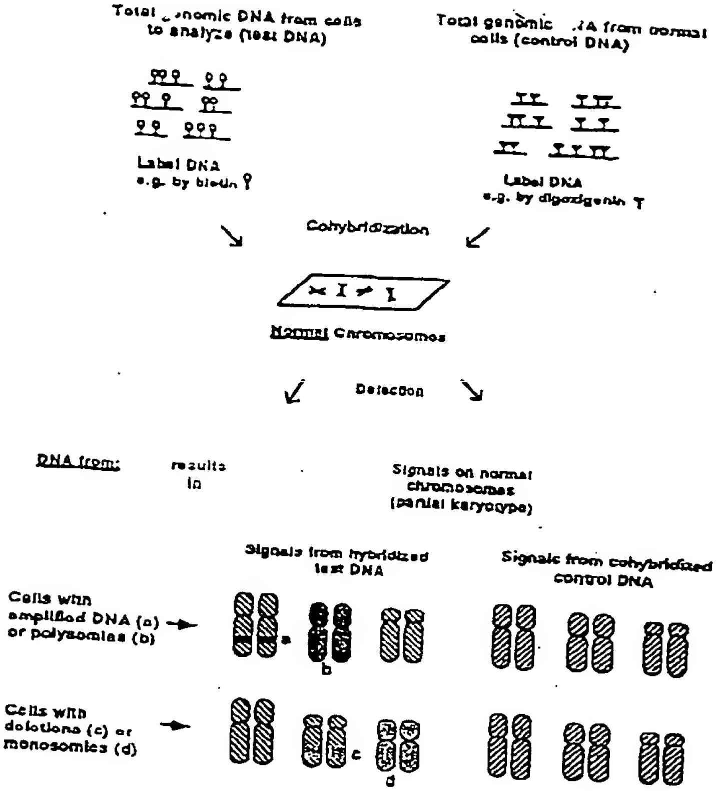

Total genomic DNA from cells to analyze (test DNA)
Total genomic DNA from normal cells (control DNA)
Label DNA e.g. by biotin
Label DNA e.g. by digoxigenin
Cohybridization
Normal Chromosomes
Detection
DNA from:
results in
Signals on normal chromosomes (partial karyotype)
Signals from hybridized test DNA
Signals from cohybridized control DNA
Cells with amplified DNA (a) or polysomies (b)
Cells with deletions (c) or monosomies (d)
a
b
c
d

Figure 6.8.
Schematic illustration of comparative genomic hybridization (CGH). Labeled genomic DNA from tissue samples to be analyzed (test DNA) and differently labeled DNA derived from normal tissue (control DNA) are hybridized simultaneously to normal metaphase chromosomes. As a result, a general background staining of all chromosomes is obtained (dashed lines). Chromosomal sequences present in additional copies within the test DNA (i.e., due to amplifications or polysomies) result in a higher staining at the corresponding chromosomal target sequences (indicated in black), as compared with the control DNA. Losses of chromosomal sequences (i.e., due to deletions or monosomies) result in a weaker staining of the corresponding target chromosomes (indicated by a lighter staining).

monly detect hybridized test and control DNA with FITC and rhodamine, respectively. However, the fluorochromes can also be used conversely without hampering the quality of the analysis.

Diagnostic variations of signal intensities in CGH can only be obtained when the signal portion of the ubiquitously occurring interspersed repetitive sequences (IRS) within DNA probes is efficiently suppressed. Suppression has to be as complete as possible in order to allow an accurate measurement of the fluores cence intensities. Standard in situ suppression hybridiza tion protocols for genomic DNA probes (Landegent e al. 1987; Lichter et al., 1988; Pinkel et al., 1988), as gen erally used for gene mapping or in clinical diagnostics, allow for some background staining through IRS. In contrast, CGH requires a rather complete suppression of IRS signals. This is achieved by using large amounts of human Cot1 DNA, representing the fast associating fraction of highly repetitive genomic DNA sequences in the suppression hybridization protocol. Since this could become very costly, the preparation of Cot1 DNA might be recommended for laboratories routinely applying CGH on a large scale.

The assessment of differences in signal intensities that would be of diagnostic value is achieved by comparing the signals from the two genomes in different colors along the chromosomes. The different possibilities to perform such a comparison are discussed in this chapter, following the CGH in situ hybridization protocol. Whereas visual inspection allows an evaluation to some extent, quantitative measurements of fluorescence intensities applying digital image analysis allow CGH at a significantly higher level of accuracy and resolution. After normalization of fluorescence intensities, the expected ratio of the signal from the test genome to the signal from the control genome is 1.0, if the chromosomal region is balanced (disomic); 0.5 when there is a deletion, monosomy, or hemizygosity; 1.5 when there are partial or complete trisomies; and so on. Correspondingly, much higher ratios are expected for higher degrees of amplification. The calculation of such ratios relies on the quantitative assessment of fluorescence intensities, which is greatly facilitated by image analysis. To obtain fluorochrome images suitable for this purpose, it is very important to standardize the image acquisition procedure. This is described later.

CGH analysis by quantitative measurements certainly puts high demands on the evaluation equipment, namely, the microscope, the camera system, and the computer software program. Therefore, the specific demands on the equipment are dealt with separately.

Laboratory Protocols

The CGH protocol is very similar to many standard FISH protocols. However, special care has to be taken at numerous points in order to obtain good CGH results. This protocol, with particular emphasis on the critical steps, is described in the following.

Preparation of Metaphase Chromosomes

PROTOCOL 6.4

Preparation of Metaphase Chromosomes from Peripheral Blood Cells

REAGENTS, BUFFERS, AND SOLUTIONS

NH_4-heparinized blood.

Culture medium: RPMI 1640, fetal calf serum (10%), L-glutamine (1%), penicillin and streptomycin (1%), phytohemagglutinin (PHA) (1.5%, dissolved in water)

Colcemid solution. Prepare stock solution of 10 μg/ml and store at −20°C.

Hypotonic solution (per liter): 3 g KCl, 0.2 g EGTA, 4.8 g HEPES, adjust to pH 7 with 1 M KOH

Fixative solution: 3 parts methanol (absolute), 1 part acetic acid (glacial). Prepare immediately before use and keep on ice.

IN ADVANCE

Precleaned microscope slides are washed in ethanol for several hours, dipped several times in water, and dried immediately before cells are dropped.

PROCEDURE

1. Add 5 ml of heparinized blood to 50 ml of culture medium.
2. Incubate at 37°C in an atmosphere of 5% CO_2 for 72 hours.
3. Add colcemid stock solution to a final concentration of 0.1 μg/ml.
4. Incubate for 20 minutes at 37°C.
5. Transfer to 50-ml tubes (polystyrol) and spin at 200 *g* for 10 minutes.
6. Remove supernatant, resuspend cell pellet in remaining medium, and add a few drops of hypotonic solution.
7. Fill tube with hypotonic solution, carefully mix, and incubate for 15 minutes at 37°C.
8. Spin at 200 *g* for 10 minutes.
9. Remove supernatant, resuspend the pellet, and add a few drops of ice-cold fixative solution.
10. Slowly add, drop by drop, 5 ml of cold fixative solution to each tube while constantly whirling the suspension.
11. Spin at 200 *g* in a cooled (4°C) centrifuge.
12. Repeat steps 9 through 10.
13. Incubate the tube on ice for 30 to 60 minutes.
14. Spin at 200 *g* in a cooled (4°C) centrifuge and repeat steps 9 through 11 at least five more times (keep tubes on ice).
15. Resuspend cells in a small volume (e.g., 0.5 to 1 ml) of fixative solution.

1. Drop the cell suspension from a distance of about 50 cm onto pretreated slides (see above). Slides should be kept in a moist environment during this procedure. Usually one drop of the cell suspension results in a sufficient amount of metaphase spreads for one CGH experiment. Check the concentration of cells with a microscope. If it is too high, add fixative solution; if it is too low, spin again and resuspend in a smaller volume. If large amounts of residual cytoplasm are still visible on the slides, repeat steps 9 through 11 several times (also see "Comments").
17. Keep the slides in a moist chamber for another 5 minutes.
18. Air dry slides.
19. Dehydrate slides in a series of 70%, 90%, 100% ethanol for 5 minutes each.
20. Air dry again and keep the slides at room temperature for 1 day.
21. Slides can either be used directly for CGH experiments within a few days or stored at −80°C for several months. To prevent water condensation, put the slides in containers sealed in plastic bags with drierite. Slowly thaw the slides before use.

COMMENTS

Following the protocol, 100 to 200 slides with metaphase spreads are obtained. For optimal CGH experiments, it is very critical to prepare reference chromosomes on slides devoid of residual cytoplasm. This seems to be crucial to obtain a homogeneous staining pattern of the chromosomes. Before using any batch for a set of experiments, it should be tested by in situ hybridization with normal genomic DNA. Furthermore, optimal spreading of the chromosomes, with few chromosome overlaps and sufficient space between metaphase spreads and interphase nuclei, is important.

Isolation of Genomic DNA

PROTOCOL 6.5

Isolation of Genomic DNA from Blood

REAGENTS, BUFFERS, AND SOLUTIONS

Lysis buffer (1 liter): 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; adjust to pH 7.4 using HCl.

SE buffer: 75 mM NaCl, 25 mM Na_2EDTA; adjust to pH 8.0 using NaOH

TE buffer: 10 mM Tris, 1 mM Na_2EDTA; adjust to pH 8.0 using HCl

Proteinase K stock solution: 20 mg/ml dissolved in 10 mM Tris-HCl (pH 7.5)

Phenol/SE, equilibrated to pH between 7.5 and 8.0. Melt approximately 200 g of crystalline phenol at 65°C and add 0.25 g of hydrochinolin. Add an equal volume of 1 M Tris-HCl (pH 8.0) and stir on a magnetic stirrer for 15 minutes.

Remove the upper, aqueous phase after separation from the lower, organic phase. Add an equal volume of SE buffer (pH 8), stir for 15 minutes, and again remove the aqueous phase after separation. Repeat this step three times, but leave the aqueous phase after the last equilibration. Keep the phenol solution at 4°C in a lighttight bottle for up to 1 month.

PROCEDURE

1. Mix 1 volume (about 5 to 10 ml) of heparinized blood and 3 volumes of lysis buffer. Shake carefully and incubate for 30 minutes on ice. Spin at 400 *g* for 10 minutes at 4°C.
2. Discard supernatant and add 1 volume of lysis buffer. Dissolve the pellet and spin at 400 *g* for 10 minutes at 4°C.
3. Discard supernatant and add 1/2 volume of SE buffer. Dissolve the pellet and spin at 400 *g* for 10 minutes at 4°C. Discard supernatant. Sediment can be stored at this point in −70°C.
4. Add 1/2 volume of SE buffer. Dissolve the pellet and add proteinase K to a final concentration of 100 μg/ml. Add sodium dodecylsulfate (SDS) to a final concentration of 1%. Mix carefully and incubate overnight at 37°C in a water bath.
5. Add 1/2 volume of SE buffer and 1 volume of equilibrated phenol/SE. Gently shake for 30 minutes and spin at 2000 *g* for 5 minutes at 10°C. Transfer upper phase in a new centrifuge tube.
6. Add 1 volume of equilibrated phenol/SE. Gently shake for 30 minutes and spin at 2000 *g* for 5 minutes at 10°C. Transfer upper phase in a new centrifuge tube.
7. Add 1/2 volume of equilibrated phenol/SE and 1/2 volume chloroform/isoamylalcohol (24:1). Shake for 30 minutes and spin at 2000 *g* for 5 minutes at 10°C.
8. Transfer upper phase in a new centrifuge tube. Add 10 ml of chloroform/isoamylalcohol (24:1). Shake for 30 minutes and spin at 2000 *g* for 5 minutes at 10°C.

9. Repeat step 8 twice.
10. Transfer upper phase in a new centrifuge tube. Add 1/10 volume 3 M sodium-acetate (pH 5.2) and 1 volume isopropanol.
11. Swirl the solution until a DNA precipitate is visible. Remove precipitate using a Pasteur pipette, the tip of which has been sealed and bent by heat. Briefly wash the DNA precipitate in 70% ethanol. Dissolve the DNA for at least 24 hours in TE buffer and store at 4°C.

If no precipitate becomes visible (i.e., if only small amounts of DNA are prepared), isolate the precipitated DNA by centrifugation at 2000 g for 10 minutes. Discard the supernatant, wash the DNA pellet with 70% ethanol, and spin again at 2000 g for 10 minutes. Remove the supernatant, dry the pellet, and finally dissolve the DNA in TE buffer.

PROTOCOL 6.6

Isolation of Genomic DNA from Solid-Tissue Samples

REAGENTS, BUFFERS, AND SOLUTIONS

Extraction buffer: 10 mM Tris-HCl (pH 8), 100 mM Na_2EDTA, 20 μg/ml pancreatic RNAase (DNAse free), 0.5% SDS

TE buffer: 10 mM Tris, 1 mM Na_2EDTA, adjust to pH 8.0 using HCl

Phenol/TE, equilibrated to pH 7.5 to 8.0. Prepare as described in protocol 6.4, but using TE buffer instead of SE buffer

PROCEDURE

1. Cut tissue sample (about 10 to 100 mg) into small pieces using a razor blade.
2. Incubate in 10 ml of extraction buffer for 1 hour at 37°C.
3. Add proteinase K to a final concentration of 100 μg/ml.
4. Incubate for 1 to 2 days at 45°C with constant shaking.
5. Add 10 ml of equilibrated phenol/TE. Gently shake for 1 hour, and then spin at 2000 g for 5 minutes at 10°C. Transfer upper phase in a new centrifuge tube.
6. Repeat step 5 twice more.
7. Add 10 ml of chloroform/isoamylalcohol (24:1). Shake for 30 minutes and spin at 2000 g for 5 minutes at 10°C. Transfer upper phase in a new centrifuge tube.
8. Repeat step 7 twice more.
9. Transfer upper phase in a new centrifuge tube. Add 1/10 volume 3 M sodium-acetate (pH 5.2) and 1 volume isopropanol.
10. Proceed as described in protocol 6.5 (step 9).

COMMENTS

The quality of the genomic probe DNA critically influences the detection sensitivity of chromosomal imbalances by CGH experiments. For example, the incorporation of modified nucleotides by nick translation (see protocol 6.8) is poor in crude DNA preparations, resulting in an irregular and speckled hybridization pattern of the metaphase chromosomes. As a result, differences in staining intensities are less pronounced. CGH can also be performed using DNA from paraffin-embedded tissue samples, as well as DNA that has been amplified by universal PCR (see protocol 6.7).

With regard to genomic control DNA, it should be noted that interindividual differences in the staining of chromosomal regions containing high proportions of repetitive sequences can occur. We have found, for example, that DNAs from different donors may vary with regard to the staining intensity of chromosome 19. It is, therefore, desirable to obtain control DNA (e.g., from peripheral blood) from the patient whose tumor DNA is being examined. If this is not possible, it is advisable to prepare a batch of control DNA derived from one especially suitable donor that can be used for a large number of CGH experiments.

Amplification of Small Amounts of Genomic DNA by the Universal Polymerase Chain Reaction (PCR)

PROTOCOL 6.7

DOP-PCR

REAGENTS, BUFFERS, AND SOLUTIONS

10× reaction buffer: 20 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl (pH 8.4), 1 mg/ml gelatin

10× nucleotide mixture: 2 mM each of dNTP (dATP, dCTP, dGTP, dTTP).

10× DOP primer: 20 μM of the oligonucleotide 5'CCGACTCGAGNNNNNNATGTGG3', (N = A, C, G, or T) (Telenius et al., 1992)

For minigels: agarose; TBE buffer—0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0; suitable size (1-kb) standard; gel loading buffer containing 0.25% bromphenol blue and 30% glycerol

DNA template. Isolate genomic DNA from small tissue samples (1 to 5 mg) as described in protocol 6.6; 1 mg of tissue results in about 1 µg of genomic DNA. For an estimation of the DNA amount obtained, put an aliquot on an ethidium bromide-stained gel (see protocol 6.7, step 4), and compare the staining of the DNA under ultraviolet light with that of a reference DNA. For protocols of DNA isolation from smaller amounts of tissue (down to only a few hundred cells) or from paraffin-embedded material, the reader is referred to the specialized literature (Speicher et al., 1995; Kawasaki, 1990).

PROCEDURE

1. Combine in a 500-µl reaction tube on ice for a total reaction volume of 50 µl:
   0.1 to 1 ng genomic DNA in a volume of 1 to 10 µl
   5 µl 10× PCR buffer
   5 µl 10× nucleotide mixture
   5 µl 10× DOP primer
   Adjusted to 50 µl with H_2O
   1.25 U Taq. polymerase (should be added last)
2. As a negative control, combine the same solutions, but without DNA.
3. Overlay reaction mixtures with 50 µl of mineral oil and transfer reaction tubes to an automated temperature-cycling machine (PCR machine).
4. For universal amplification of the genomic DNA, the following temperatures and times are applied.
   Initial denaturation of the DNA template by heating at 94°C for 10 minutes.
   Five cycles of (low-stringency) amplification at 94°C, 1 minute; 30°C, 1.5 minutes; 30 to 72°C transition, 3 minutes; 72°C, 3 minutes
   35 cycles of (high-stringency) amplification at 94°C, 1 minute; 62°C, 1 minute; 72°C, 3 minutes (with an addition of 1 s/cycle; the final extension temperature of 72°C is applied for 10 minutes)
5. Control the PCR reaction by agarose gel electrophoresis. As a size marker, use, for example, a 1-kb ladder. Run the gel in 1× TBE buffer for 30 minutes at 100 volts, stain with ethidium bromide, and photograph as described in protocol 6.8, step 4. A smear of DNA, ranging in size from about 200 to 2000 bp, should be visible. No smear should be visible in the negative control.
6. Label the amplified DNA by nick translation as described in protocol 6.8 and use as a probe in a CGH experiment. Alternatively, the DNA can be labeled by adding modified nucleotides in the PCR reaction.

COMMENTS

The preceding protocol, termed DOP-PCR (because a *degenerated oligonucleotide primer* is used), was first developed and described by Telenius et al. (1992). Using DOP-PCR amplification, DNA isolated from only a few cells (about 5 to 500) is sufficient for the analysis of chromosomal imbalances by CGH (Speicher et al., 1993). As an alternative, sequence independent-amplification (SIA) could be used (Bohlander et al., 1992). It should be noted that PCR, in general, is a very sensitive approach and prone to artifacts by DNA contamination. To avoid this, only autoclaved and ultrapure materials and reagents should be used and gloves should be worn. PCR reaction mixtures and PCR products must be prepared in separate rooms. Finally, a negative control should always be included in the experiments (see above).

Probe labeling

Nick translation is the most frequently applied method for labeling DNA probes to be used in fluorescence in situ hybridization experiments. Although the labeling efficiencies of nick translation and primer extension (Feinberg and Vogelstein, 1984) are comparable, we prefer nick translation because, by varying the DNase concentration in the reaction solution, it permits an easy adjustment of the probe size. For optimum CGH conditions, it should be between 500 and 1000 nucleotides long. Our protocol is based on the work of Langer et al. (1981). The labeling is described with biotin and digoxigenin, as these reporter molecules are the most popular owing to their sensitivity and commercial availability. In our laboratory, biotin is used for the labeling of the test DNA and digoxigenin for the labeling of the control DNA. Alternatively, directly coupled fluorochromes could be used to substitute biotin-dUTP.

PROTOCOL 6.8

Biotinylation of the Test DNA by Nick Translation

REAGENTS, BUFFERS, AND SOLUTIONS

Solution of the test DNA to be labeled

10× reaction buffer containing 0.5 M Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin

0.1 M β-mercaptoethanol. Add 0.1 ml of β-mercaptoethanol to 14.4 ml of double-distilled water.

10× nucleotide stock containing 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM biotin-16-dUTP, and 0.12 mM dTTP (alternatively, other biotinylated dUTP derivatives can be used)

Escherichia coli DNA polymerase I

DNase I solution, 3 mg in 1 ml 0.15 M NaCl, 50% glycerol

Column buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% SDS

For spin columns: Sephadex G50 (medium), 1-ml syringes, glass wool

For minigels: agarose, TBE buffer, suitable size (1-kb) standard, gel loading buffer as described in protocol 6.7

EQUIPMENT

Water bath, which can be cooled to 15°C

Gel electrophoresis to analyze the reaction product

PROCEDURE

(For a 100-μl reaction allowing the labeling of 2 μg of probe DNA)

1. Combine 2 μg of probe DNA, 10 μl of 10× reaction buffer, 10 μl of β-mercaptoethanol, 10 μl of nucleotide stock, 20 U of DNA polymerase I, and the tested volume of a 1:1000 dilution of DNase I. Adjust to 100 μl with double-distilled water (enzymes should be added last).
2. Incubate for 2 hours at 15°C.
3. Put the reaction solution on ice. It should be kept on ice until the actual size of the reaction product has been determined.
4. Check the length of the probe molecules by gel electrophoresis. Take 10 μl out of the reaction solution, add gel loading buffer to the aliquot, and denature the solution by boiling in a water bath for 2 to 3 minutes. After another 3 minutes on ice, the aliquot is loaded on a standard 1 to 2% agarose minigel with a suitably sized marker and run at 15 V/cm for 30 minutes. For the visualization of the DNA, stain the gel in 0.5 μg/ml of ethidium bromide and take photographs under ultraviolet illumination.
5. For optimum hybridization conditions, the probe (which is visible as a smear) should be between 500 and 1000 kb in length. Depending on the result of the gel, proceed as follows:
    a. If the probe size is within the desired range, proceed to step 6.
    b. If the probe size is larger, add more DNase I, incubate at 15°C (usually higher amounts of DNase I are added for another 30 minutes), and repeat step 4.
    c. If the probe is not or is almost not digested, purify the probe and start the nick translation again.
    d. If part of the probe is less than 100 kb in length, start the reaction again using less DNase I.
6. To inactivate the DNase, 2 μl of 0.5 M EDTA (final concentration 10 mM) and 1 μl of 10% SDS (final concentration 0.1%) are added and the reaction mix is heated at 68°C for 10 minutes.
7. Unincorporated nucleotides are separated from the labeled probe by gel filtration using a spin column as follows:
    a. Pack a 1-ml syringe with silanized glass wool up to the 0.2-ml mark and with buffered Sephadex G50 to the 1-ml mark. Put the column in a 15-ml tube and spin it at 2000 *g* for 6 minutes at room temperature.
    b. Remove flowthrough, fill again, and repeat centrifugations until the resin is tightly packed to the 1-ml mark. Add 100 μl of column buffer and spin again at 2000 *g* for 6 minutes. Repeat this washing step three times. After the last washing step, make sure that the volume of the flowthrough equals the volume of the loading buffer, that is, 100 μl.
    c. Before centrifuging the probe solution, put a small reaction tube in the 15-ml tube underneath the syringe. Load the probe solution into the column and spin as before.

The flowthrough is collected in the reaction tube and contains the labeled probe at a concentration of approximately 20 ng/µl. It is ready for use in a CGH experiment, or it can be stored at −20°C.

PROTOCOL 6.9

Digoxigenin Labeling of the Control DNA by Nick Translation

Digoxigenin labeling of DNA probes is carried out in exactly the same manner as biotinylation by nick translation with one exception: the 10× nucleotide stock contains 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.125 mM digoxigenin-11-dUTP, and 0.375 mM dTTP.

Similarly, directly fluorochrome-labeled nucleotides can be used instead of digoxigenin-11-dUTP.

COMMENT

If DNase I has to be added several times after the first digestion step, this most often is the result of protein contamination of the DNA. In such cases, an additional phenol-extraction step should be performed (protocol 6.5, steps 5 through 8). When DOP-PCR products are used for labeling, DNAse I concentration and digestion times need to be adjusted according to the length of the PCR products. DNase I concentrations below 10 U/100 µl and digestion times of 1 hour are not unusual and will result in an efficient labeling of the DNA. After the preparation of new reagents or when no hybridization signals are obtained, it is advisable to check the incorporation of the biotin- or digoxigenin-labeled nucleotides. This can be achieved with a dot blot assay, which is a simple colorimetric assay (protocol 6.10).

PROTOCOL 6.10

Dot-Blot Assay to Test Labeling

REAGENTS, BUFFERS, AND SOLUTIONS

DNA dilution buffer: 0.1 mg/ml sheared salmon sperm DNA, 6 × SSC (1 × SSC: 0.15M NaCl, 0.015 sodium citrate, pH 7.0)

Series of dilution of biotinylated standard DNA (commercially available standard or previously used, well-labeled probe) in DNA dilution buffer, for example, 1, 3, 10, 20 pg/µl standard DNA

AP 7.5 buffer: 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 2 mM $MgCl_2$

AP 9.5 buffer: 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$

Blocking buffer: 3% BSA in AP 7.5 buffer

TE buffer: 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA

75 mg/ml nitroblue tetrazolium (NBT) in 70% dimethylformamide and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylformamide

EQUIPMENT

Nitrocellulose filter

PROCEDURE

1. Put 1-µl aliquots of the different dilutions of standard DNA and, in parallel, 1-µl aliquots of the same concentrations of test DNA on a nitrocellulose filter.
2. Bake the filter at 80°C for 1 hour.
3. Place the filter in a dish and wash with AP 7.5 buffer for 1 minute at room temperature.
4. Seal the filter in a plastic bag, together with 10 ml of blocking solution. Incubate at 37°C for 30 minutes.
5. Open the bag at one end, remove the blocking solution, and add a freshly prepared solution of streptavidin-conjugated alkaline phosphatase (diluted to a concentration of 1 µg/ml in AP 7.5), seal the bag again, and incubate at 37°C for 30 minutes.
6. Take the filter out of the plastic bag and wash in a dish with AP 7.5 buffer (twice for 5 minutes at room temperature), followed by AP 9.5 buffer (10 minutes at room temperature).
7. Seal the filter again in a plastic bag, together with the developing solution: 33 µl of NBT added to 5 ml of AP 9.5 buffer. After careful mixing (do not vortex!), 25 µl of BCIP is added; the resulting solution is again mixed gently. Incubate at 37°C until color development is appropriate. Usually 15 to 60 minutes are sufficient.
8. Take the filter out of the plastic bag and wash it in a dish with TE buffer to stop the color reaction.

9. After air drying, the assay can be evaluated: the color intensities of the test and control DNA should be comparable. For optimal hybridization results, even the 1-pg signal should be visible.

When the dot-blot assay is carried out to check the digoxigenin labeling, an antidigoxigenin-labeled alkaline phosphatase is used (step 5).

Fluorescence in situ hybridization and detection

For CGH, genomic DNAs containing abundant amounts of interspersed repetitive sequences (IRS) are used as probes. For the suppression of unspecific hybridization signals arising from these sequences, the DNA probes are combined with an excess of unlabeled Cot1 DNA and a preannealing step is performed after denaturation of the probe solution (Pinkel et al., 1988; Lichter et al., 1988). A protocol is given for a hybridization volume of 12 µl that is sufficient to hybridize an area of 18 by 18 mm on a glass slide.

PROTOCOL 6.11

Probe Mixture and Denaturation

REAGENTS, BUFFERS, AND SOLUTIONS

3 M sodium acetate, pH 5.2

Deionize formamide (molecular biology grade) using ion exchange resin. The conductivity of the solution should be below 100 µSiemens.

Hybridization buffer: 4 × SSC, 20% dextran sulfate. Prepare 20 × SSC and 50% dextran sulfate solutions. After careful dissolving, autoclave the dextran sulfate solution or filter it through a nitrocellulose filter. Mix 200 µl of 20 × SSC, 400 µl of 50% dextrane sulfate, and 400 µl of double-distilled water. Store at 4°C until use.

PROCEDURE

1. Combine 1 µg each of labeled test and control DNA and 50 to 100 µg of human Cot1 DNA. Precipitate by adding 1/20 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. Mix well and incubate at −70°C for 30 minutes.
2. Spin in an Eppendorf centrifuge at 12,000 rpm for 10 minutes at 4°C. Discard the supernatant, wash the pellet by adding 500 µl of 70% ethanol, and spin again (12,000 rpm, 10 minutes, 4°C). Discard the supernatant and lyophilize.
3. Resuspend in 6 µl of deionized formamide by vortexing for more than 30 minutes at room temperature.
4. Add 6 µl of hybridization buffer and again vortex for more than 30 minutes. At this step, proceed with denaturation of the DNA on the slide (protocol 6.12).
5. Denature DNA at 75°C for 5 minutes, followed by incubation on ice for another 5 minutes and preannealing of the probe solution at 37°C for 20 to 30 minutes.

PROTOCOL 6.12

Denaturation of Chromosomal DNA on Slides

REAGENTS, BUFFERS, AND SOLUTIONS

Denaturation solution: 70% deionized formamide, 2 × SSC, 50 mM sodium phosphate, adjust to pH 7.0 by adding HCl

Ice-cold ethanol: 70%, 90%, and 100%

PROCEDURE

1. Select appropriate area on the slides for hybridization and mark them from underneath with a diamond pen.
2. Incubate slides in an oven at 60°C before denaturation. This will prevent dropping of the temperature in the denaturation solution when the slides are added.
3. Fill a Coplin jar with the denaturation solution and put it in a water bath heated to 70°C. Before denaturation, check the temperature of the denaturation solution with a thermometer inside the jar. This is a critical step! For good results, the temperature should be at 70°C.
4. Transfer the prewarmed slides (not more than three at a time) into the Coplin jar with the denaturation solution for exactly 2 minutes.
5. Immediately transfer the slides into Coplin jars with 70%, 90%, and 100% ethanol (on ice) for 5 minutes each.
6. After air drying, the slides are ready for hybridization.

When denaturation is not sufficient, efficient hybridization cannot be performed. Overly denatured chromosomes usually look fuzzy when counterstained with DAPI, or may even disintegrate.

PROTOCOL 6.13

Hybridization

PROCEDURE

1. Apply 12 μl of hybridization mixture with the denatured and preannealed probe to the denatured chromosomes on the slides.
2. Put an 18- by 18-mm cover glass on top of the hybridization droplet. Take care not to trap air bubbles.
3. Seal the edges of the cover glass with rubber cement and put the slides in a wet chamber. Incubate for 48 hours at 37°C.

After hybridization, detection of the probe sequences on the slides is achieved using protocol 6.14.

PROTOCOL 6.14

Detection

REAGENTS, BUFFERS, AND SOLUTIONS

Wash solution A: 50% formamide (inexpensive grade), 2 × SSC, pH 7.0
Wash solution B: 0.1 × SSC, pH 7.0
Wash solution C: 4 × SSC, 0.1% Tween 20, pH 7.0
Wash solution D: 2 × SSC, 0.05% Tween 20
Blocking solution: 3% BSA, 4 × SSC, 0.1% Tween 20
Detection buffer: 1% BSA, 4 × SSC, 0.1% Tween 20
Antifade mounting solution. Such solutions considerably decrease the bleaching of the illuminated fluorochromes. Several commercial reagents are available; of these, Vecta Shield (Vector Laboratories) has proved to be very efficient. Alternatively, DABCO antifading medium can be prepared: 0.233 g of DABCO (1,4-diazabicyclo-2.2.2-octane), 20 mM Tris-HCl, pH 8.0, 90% glycerol. Store this solution in the dark at 4°C.

PROCEDURE

During the entire protocol, the slides must not become completely dry.

1. Prewarm washing solutions A and B in a 42°C water bath and a 60°C water bath respectively.
2. After taking the slides out of the wet chamber, carefully remove the rubber cement using a forceps.
3. Transfer the slides into a Coplin jar containing washing solution A prewarmed to 42°C, and agitate in a shaking water bath for 10 minutes until the cover glasses come off. Transfer the slides to another jar with washing solution A and agitate for 5 minutes. Change washing solution A twice more and shake for 5 minutes each time.
4. Transfer the slides to a Coplin jar containing prewarmed (60°C) washing solution B and wash for 5 minutes. Change the solution twice, each time washing for 5 minutes.
5. Take the slides out of the jar, drain them, and apply 200 μl of blocking solution.
6. Cover with a 22- by 40-mm cover glass, put the slides in a wet chamber, and incubate at 37°C for at least 30 minutes.
7. Let the cover glass slide off each slide, drain any excess fluid, and add 200 μl of detection solution containing the 5 μg/ml of fluorescein-conjugated avidin and 6 μg/ml of rhodamine-conjugated antidigoxigenin. Incubate in a wet chamber at 37°C for 30 minutes. All subsequent steps should be carried out in dark Coplin jars (e.g., wrapped with aluminum foil).
8. Again let the cover glass slide off, transfer the slides into washing solution C, and wash for 3 × 5 minutes at 42°C (shaking water bath).
9. Transfer the slides into a Coplin jar containing counterstaining solution (2 × SSC, 200 ng/ml DAPI) and agitate at room temperature for 20 minutes.
10. Transfer the slides into a jar with washing solution D and incubate for 1 to 2 minutes at room temperature.
11. Take each slide out of the Coplin jar, add 20 to 30 μl of antifade solution, and cover with a 22- by 40-mm cover glass. Put the slides in suitable boxes, which should be kept at 4°C for long-term storage.

COMMENTS

In contrast to the hybridization conditions given above, lower concentrations of test, control, and Cot1 DNA have been used successfully by other groups (Kallioniemi et al., 1994; Ried et al., 1994). Under these conditions, the hybridization times were extended to 3 to 5 days. However, in our hands, a more homogenous staining pattern was achieved using the protocol described.

Image Acquisition

Fluorescence signals are visualized applying conventional epifluorescence microscopy and images are acquired using a suitable camera system. For optimum illumination, the mercury lamp should be precisely adjusted by aligning the focused lamp image with the lamp mirror image. The light source should be centered in order to facilitate a homogeneous illumination. Contrast within the image is increased by closing the field diaphragm to the border of the image acquisition area. DAPI, FITC, and rhodamine images are visualized subsequently, using filter sets selective for each of the fluorochromes (single bandpass filters).

At this step, in many instances, gross genomic imbalances (e.g., high-level DNA amplifications or gains and losses of entire chromosomes) of the tumor can be readily detected by visual inspection. Furthermore, a rough assessment of the quality of the hybridization experiment can be made according to the following criteria.

- When tumor or test DNA is derived from a male, a weaker staining of the X chromosome should be visible.
- Background staining outside the areas occupied by chromosomes or nuclei should be minimal.

For a comprehensive assessment of all chromosomal imbalances present in the tumor genome, a quantitative measurement and analysis of fluorescence intensities are necessary. For this purpose, digitized images have to be obtained using a sensitive camera, such as a cooled CCD (charged-coupled device) camera (Hiraoka et al., 1987). Exposure times for each fluorochrome have to be chosen that result in maximum pixel values equaling half of the dynamic range of the camera. All optical settings, as well as exposure times, have to be kept constant for images obtained in a series of metaphase spreads acquired for a single case. In our experience, using a cooled CCD camera, exposure times are in the range of 3 to 5 seconds for FITC and 0.5 to 2 seconds for rhodamine images. For the quantitative evaluation, special care has to be taken to select metaphase spreads with no or very few overlapping chromosomes.

Evaluation of CGH Experiments

Since CGH is a fairly new procedure, the modes of evaluation are still the subject of intensive discussion. Future scientific meetings will be necessary to attain agreement on generally accepted criteria for standardized evaluation procedures. For the current status of this discussion, the reader is referred to the literature (Kallioniemi et al., 1993; Du Manoir et al., 1994; Lundsteen et al., 1994; Piper et al., 1994). The evaluation can be done on different levels of sophistication, which, in general, correlate with the level of precision regarding the obtained data. The investigator should be aware of the differences in the accuracy of the evaluation obtained by visual inspection and by quantitative measurements. Whereas for some applications, such as screening for possible amplified DNA sequences in a tumor, visual inspection is sufficient, other applications require an analysis of chromosomal imbalances that is as complete as possible.

In a good CGH experiment, imbalances resulting from gross changes of chromosomal material are readily visible under a fluorescence microscope. For example, amplifications are seen as strong dotlike signals (see Figs. 6.7 and 6.12), and the complete lack of a chromosomal region is immediately visible, since the corresponding segment on the normal metaphase spread will not become painted. As fluorescence signals will fade during longer examinations, it is more convenient to acquire digitized images of the FITC and rhodamine fluorescence and to perform the visual comparison of these images at the monitor (compare Fig. 6.9 (color plate 10), panels C and D). In a comparative study, it was shown that it is possible to analyze complex chromosomal imbalances by such a visual inspection of images (Du Manoir et al., 1993). However, the procedure is very time consuming and subtle imbalances could remain unidentified.

Visual inspection of images can be facilitated by applying variable thresholding or pseudocolorization to the test DNA image in order to visualize intensity differences for the observer's eye. However, it should be noted that these procedures are not equivalent to a real quantitative measurement, and certainly are not comparable to the calculation of the ratio between test and control DNA signals.

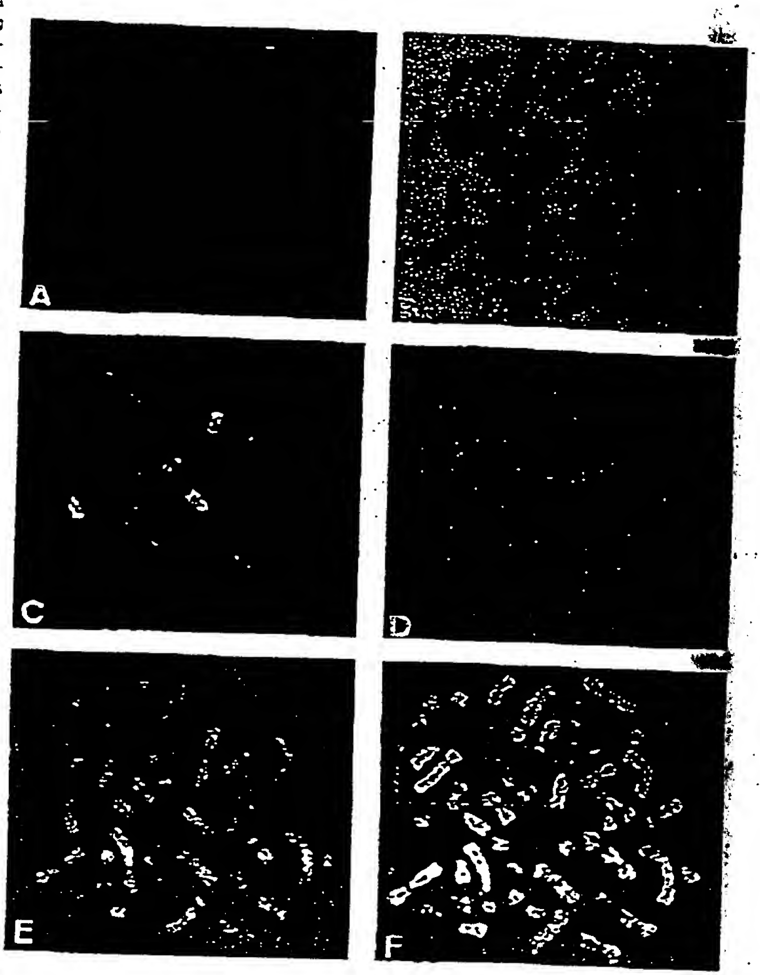
Digitized images obtained for hybridized test and control DNA can also be overlayed electronically, resulting in an image in which either mixed colors (Fig. 6.9, panel E) or only the most intensive of the two colors appears. Although this provides another nice illustration of the signal variations, it usually does not improve the ability to assess imbalances by visual inspection.

Quantitative measurements of fluorescence intensities clearly are required for an accurate CGH analysis of low copy number changes, especially if they are not present in the vast majority of cells. Furthermore, this type of analysis is indispensable in complex cases harboring multiple DNA gains and losses.

Several software applications for quantitative CGH evaluation are currently being developed using different informatic strategies. However, several requirements are crucial to a reliable evaluation, including:

1. The definition of objective criteria for the selection of CGH preparations adequate for quantitative analysis.
2. Chromosome segmentation.
3. Fluorescence background estimation.
4. Criteria for the definition of the central value in-

Figure 6.9. Detection of chromosomal imbalances by CGH and digital image analysis in a prostate tumor. (A) CCD image of a normal metaphase spread counterstained with DAPI to allow chromosome identification. (B) Digital inversion of the image shown in A. This procedure provides a banding pattern resembling classical G-banding, thus facilitating identification of the chromosomes. (C) Hybridization pattern of biotin-labeled genomic DNA derived from prostate tumor cells. The hybridized tumor DNA was detected via FITC. Using an appropriate single bandpass filter, a CCD image of only the FITC fluorescence staining was obtained. This gray-scale image was then digitally pseudocolored in green. Note the differences in the staining intensities among the individual chromosome arms. (D) Hybridization pattern of the control DNA labeled with digoxigenin and detected via rhodamine. The image was obtained as indicated in C, but pseudocolored in red. (E) Mixed-color image obtained by digital overlaying of the images shown in C and D. Greenish colors indicate chromosomal sequences that are overrepresented and reddish color sequences that are underrepresented in the tumor cells. (F) Chromosomal gains and losses as indicated by a three-color lookup table in a ratio image. Gains are indicated in green, losses are indicated in red, and the normal chromosomal complement is indicated in white. In principle, this image represents a transformation of the intensity ratio profile (see Fig. 6.11) into three colors (color plate 10).



dicative for a balanced representation of a specific chromosomal region.

5. The definition of thresholds for unbalanced segments present in lower or higher copy numbers.

In the following, the principles of one specialized software application (Du Manoir et al., 1994) are briefly outlined. After selecting suitable images and segmentation, chromosomes and their orientations are identified interactively (see above). Fluorescence intensities are then measured for the FITC and rhodamine images along the axes of individual chromosomes pixel by pixel, where each value represents the mean of the values perpendicular to the chromosome axis. The ratio of the fluorescence intensities is calculated and a "ratio profile" along individual chromosomes thereby generated. Such a ratio can be transformed into a three-color lockup table, where different colors are assigned to over- or underrepresented, as well as balanced, chromosomal sequences [see Figs. 6.9F and 6.10 (color plate 11)].

Owing to slight variations in hybridization quality among different metaphase spreads, the averaging of these ratio profiles over several metaphase spreads (usually 7 to 10) is necessary to detect reliably imbalances involving smaller chromosomal regions (see Fig. 6.11). For the detection of such imbalances, a high sensitivity is critical. This parameter depends largely on the threshold criteria selected; in our laboratory, a fixed range threshold (fixed ratio values defining overrepresentation [e.g., 1.25] and underrepresentation [e.g., 0.75]) has been extensively tested. By definition, with these thresholds, aberrations should be detected if they are present in more than 50 percent of cells. Copy number changes in lower proportions of cells cannot be detected, as the ratio values will not reach the thresholds. An alternative is statistical thresholds based on the calculation of the variability of the average ratio for balanced chromosomes (Du Manoir et al., 1994). Using these thresholds, the normal range can be very narrow in the case of a high-quality CGH preparation, resulting in a considerable increase in sensitivity.

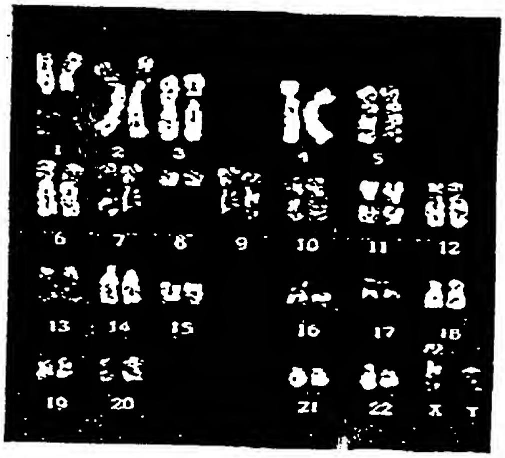


Figure 6.10.
Karyogram of the metaphase spread shown in Fig. 6.9F. The chromosomal gains (green) and losses (red) in this tumor can be recognized in a comprehensive manner. Note the high consistency of the ratios for both chromosome homologs (color plate 11).

Equipment

Microscope

For CGH analysis, an epifluorescence microscope suitable for research purposes is required. Particular attention should be paid to the availability of a stable object table, which is essential for the subsequent acquisition of images from the same metaphase spread. All major suppliers provide good-quality devices sufficient for CGH applications. Depending on the microscope type, a 100-watt mercury lamp may be necessary in order to obtain sufficiently intense and homogeneous illumination of the optical field. Use of a field diaphragm in the excitation pathway is desirable to reduce the blurring and increase the contrast within the images. In our experience, a 63× objective (ultraviolet-transparent, plan) is most useful for evaluation by eye and image acquisition in CGH. Metaphase spreads are sufficiently large for a detailed analysis of small chromosomal subregions, and yet are small enough to fit within 512- by 512-pixel margins required by most image-analysis software. Image acquisition is performed using narrow bandpass filters selective for the fluorochromes used. As several images are taken consecutively, a minimal pixel shift should be the aim. This might be achieved by a fixed dichroic and triple emission filter combined with a moving excitation filter.

Camera

Currently, high-quality, black-and-white cooled CCD cameras are used by various groups developing CGH. It is not clear whether a color CCD camera has any advantage for this application, as only the intensity of the fluorescence needs to be measured. Since spectral separation is not optimal in any type of color CCD, it may even be inferior to black-and-white systems for CGH. Essential requirements are a very homogeneous camera target response and minimal shading. As only a high sensitivity, not a high dynamic range, is required for CGH, even lower-quality cameras may be adequate.

Software

The basic ideas behind software programs designed for quantitative CGH image analysis are outlined above.

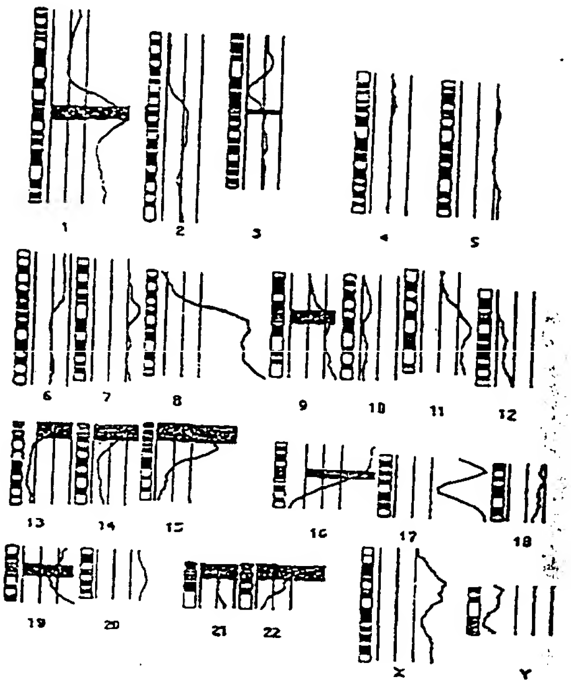


Figure 6.11.
Average green-to-red fluorescence ratio profile after hybridization with DNA from the prostate cancer case shown in Figs. 6.9 and 6.10. The ratios are plotted along each single chromosome (mean of 10 metaphase spreads analyzed). The mean intensity ratios (central line) and the thresholds for overrepresentation (right line) and underrepresentation (left line) are shown. These thresholds were calculated on the assumption that at least 50 percent of the cells carry the respective chromosomal imbalance.

Several requirements should be met by such software. Quality criteria for the selection of images, and, even more important, for the rejection of inadequate images, need to be included. Furthermore, the program should be capable of segmenting chromosomes in metaphase spreads. Most nonoverlapping chromosomes of a given metaphase spread should be assessed in a quantitative evaluation. For an accurate diagnosis of over- or underrepresentations of chromosomal material in small genomic regions, the possibility for averaging values from several metaphase spreads should be included in the program. For easy handling, minimal user interactions and help with chromosome identification would be desirable. Currently, such software is becoming commercially available.

Troubleshooting

After hybridization, a bright fluorescent painting of interphase cells and metaphase spreads should be visible under an epifluorescence microscope. It is important to note that gross aberrations, such as gains or losses of a whole chromosome or amplifications, can be detected without any equipment for digitized image acquisition or analysis. If, for example, hybridization of a male DNA does not result in a markedly weaker staining of the X chromosome(s), this indicates an inadequate quality of the hybridization experiment. Usually this finding is accompanied by other hallmarks of poor quality, such as a speckled, inhomogeneous staining pattern or high background fluorescence, which both may considerably disturb the quantitative image analysis by causing a great deal of variation within the ratio profiles.

Poor hybridization results can be attributed to several causes. In our experience, the most important factor in a good hybridization experiment is the careful preparation of metaphase spreads. Therefore, every new batch needs to be tested. If no good hybridization results are obtained, the whole batch must be discarded and a new slide-preparation procedure started. Although impaired hybridization conditions are mainly the result of residual cell debris on the slides, proteolytic digestion is not useful in achieving high-quality CGH experiments. Although it will improve the hybridization pattern on suboptimal slides, the results are considerably worse than those obtained on optimum slides without any pretreatment. Other important causes for granular and inhomogeneous staining patterns are probe DNA preparations that are contaminated with high amounts of protein or inappropriate sizes of the labeled probe fragments after nick

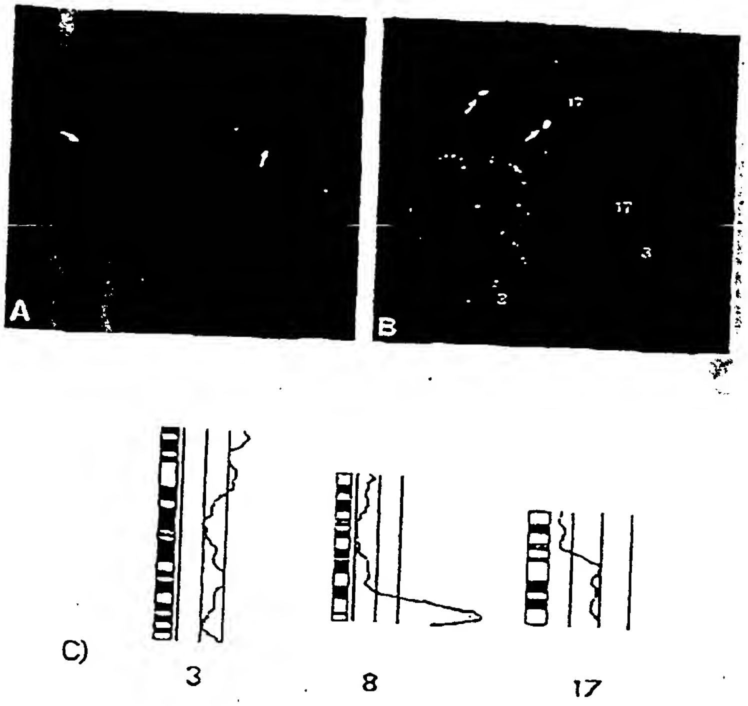


Figure 6.12. Detection of amplified sequences by CGH. (A) Direct photograph of a metaphase spread that was hybridized with DNA from a medulloblastoma containing an amplification on chromosome 8q. Because only a few tumor cells were available for this analysis, the tumor DNA was amplified by a universal polymerase chain reaction (DOP-PCR; see text). The amplification on 8q is clearly visible, even by direct visual inspection through the microscope. (B) CCD image of a metaphase spread after hybridization of the same tumor DNA as indicated in A. In addition to the detection of the amplification signals on chromosome 8q (arrows), an overrepresentation of chromosome 3p and an underrepresentation on 17p also become visible when the CCD camera is used. (C) Average ratio profiles (obtained from 10 metaphases) of the chromosomes affected in the medulloblastoma shown in A and B (color plate 12).

translation. Fragments that are too large typically result in a starry background outside of the chromosomes. In contrast, if the probe size is too small, a homogeneous background distributed over all chromosomes may be seen. This may also lead to a homogeneously strong staining pattern in regions that, in fact, are over- or underrepresented in the tumor genome. Of course, this problem can be easily overcome by checking the probe size after each labeling procedure.

In contrast to conventional FISH experiments, strong fluorescence on the chromosomes and little or no fluorescence in the areas where no chromosomes or nuclei are located do not guarantee a hybridization experiment useful for a CGH analysis, as even in the case of bright and smooth staining, evaluation of the experiment can be severely impaired. This may be the result of (1) insufficient suppression of repetitive sequences or (2) inhomogeneous illumination of the optical field. Insufficient suppression may lead to incorrect measurements of ratios in chromosomal regions with a high content of repetitive sequences that vary considerably among individuals, such as chromosome 19. Strong staining of the heterochromatin blocks on chromosomes 1, 9, 16, and 19 can indicate poor suppression. An increased amount of the Cot1 DNA fraction and longer preannealing times (the latter being less effective) can be used to achieve adequate suppression of signals in these regions of the genome. Good suppression will result in very low FITC and rhodamine fluorescence intensities of the heterochromatic chromosome regions. Thus minute absolute variations of fluorescence intensities may cause gross ratio variations within these chromosomal regions. Consequently, they are excluded from evaluation.

Inhomogeneous illumination of the optical field leads to considerable regional variations within the ratio image that render a quantitative evaluation of the CGH experiment completely impossible (see, e.g., Du Manoir et al., 1994). To overcome this problem, the light source of the microscope has to be carefully centered.

Since a wide variation in chromosome length may considerably impair the spatial resolution of the averaged ratio profiles, chromosomes of similar lengths are required.

It should be noted that, apart from these experimental conditions, the validity of a CGH experiment critically depends on the percentage of cells carrying chromosomal imbalances. If no gains or losses of chromosomes are detected, this could be due to a low proportion of tumor cells within the sample. Therefore, adequate information from the histological laboratory is crucial to any type of CGH analysis.

Future Prospects

Methodological developments

Comparative genomic hybridization combined with ratio imaging analysis allows the comprehensive assessment of genetic imbalances in any type of cell or tissue, many of which have not been accessible to a cytogenetic analysis using other methods.

Several groups are developing software programs for quantitative CGH evaluation. In most of these programs, the entire chromosome length is applied as a parameter to calculate the subchromosomal position of a signal or a signal variation without reference to banding information. Thus these applications are based on the assumption that there is a linear relationship of the distribution of chromosomal bands in different states of chromosome condensation. However, this is clearly not the case, as it is well known that G- and R-bands condense to different degrees. Therefore, it will be important to know the exact distribution of G- and R-bands for a chromosome in various states of condensation.

Currently, the chromosome ideograms defined by the International System for Human Cytogenetic Nomenclature (Harnden et al., 1985) (see also Chap. 12 in this book) are compared with the profile of a particular chromosome in order to assign profile shifts to chromosomal bands. However, these ideograms are not based on band measurements. Thus it will be important to define standardized ideograms with accurately assessed band distributions. A recent publication by Francke (1994) presents such schematic drawings, which are based on measurements of the lengths of chromosomal bands. Because of the differential condensation of chromatin, it will be helpful to define two or three such ideograms for different decondensation states of each chromosome. For example, for human chromosome 1, three banding schemes could be generated for the overall length of 10 µm (average metaphase chromosomes), 15 µm (extended metaphase chromosomes), and 20 µm (approximate prometaphase chromosomes). The standardization of such ideograms certainly is inevitable. Future nomenclature systems likely will deal with these requirements.

Alternatively, combining CGH with simultaneous high-quality banding will be of great benefit, as it should allow an improvement in the speed and resolution of CGH evaluations. However, based on the specific needs of the method, this banding should be detected in a mode that allows clear distinction from the fluorochromes used for test and control DNA. In addition, the procedure must not result in a band-specific loss of chromosomal DNA. Posthybridization banding by DAPI is often used, and a recent study has demonstrated the use of DAPI banding for semiautomated karyotyping within CGH analyses (Lundsteen et al., 1994).

Applications

Although it might be possible to use CGH for evolutionary studies involving species comparisons, at present, it is considered mainly a useful tool for tumor cytogenetics. This is especially true for the analysis of solid tumors, where chromosome banding is often difficult, or even impossible, to perform. In the few CGH papers published so far, numerous new chromosomal imbalances have been described (Kallioniemi et al., 1993; Mohamed et al., 1993; Suijkerbuijk et al., 1994). Accordingly, it is anticipated that CGH studies will result in a large set of new data regarding recurrent chromosomal aberrations in solid tumors, considerably extending the field of tumor cytogenetics. However, since CGH can only detect over- or underrepresentations of chromosomal regions, balanced rearrangements—such as many translocations and inversions specific for certain hematological malignancies—would escape the analysis. The recurrent genomic alterations detected by CGH are likely to indicate the chromosomal regions where genes relevant for tumor development or progression are localized. In this context, CGH will likely become an important method for the detection of two types of genes: proto-oncogenes, which are amplified in certain tumors, and tumor suppressor genes, one copy of which is often deleted. In contrast, chimeric genes resulting from the fusion of genes or parts thereof, which are far apart in the normal genome, are frequently found as a product of balanced rearrangements, and, therefore, they are less likely to be detected by CGH analyses.

The comparison of CGH and G-banding analysis in leukemias has revealed discrepancies in a large number of cases, indicating that karyotypes obtained by G-banding analysis may not reflect the clonal distribution of the leukemia cells in vivo (Bentz et al., 1993). Thus CGH is likely to be a powerful method for the rapid and accurate detection of chromosomal imbalances not only in solid tumors but in hematological malignancies as well.

To date, CGH has been a research tool greatly facilitating the cytogenetic analysis of many tumors. Since it will contribute to the identification of genomic regions frequently imbalanced in certain tumor types, CGH data will lead to the inclusion of DNA probes and probe sets in analyses of the corresponding tumor type by various other methods, such as interphase cytogenetics. Furthermore, it is a rapid strategy for screening a tumor genome for amplifications and determining their chromosomal localizations. Thus other methods can be applied in a very specific fashion, testing only genes mapping to the respective chromosomal loci. This provides a considerable improvement on the conventional method, in which large batteries of probes are screened, and success depends on the availability of suitable candidate probes.

It is, however, still difficult to predict whether CGH will be used in a routine diagnostic setting. As it is a technically demanding method, it will be used as a routine tool only if it exhibits clear advantages for certain applications. One area should be mentioned that might gain considerable importance for routine diagnostics. It is possible to combine CGH with a universal PCR procedure as described in this chapter. Thus chromosomal imbalances can be obtained even in small samples containing very few cells, such as provided by the routine pathology laboratory [see Fig. 6.12 (color plate 12) and Speicher et al., 1993]. Since such tissue samples would not be sufficient for a comprehensive analysis by conventional interphase cytogenetics, this might become a routine application for CGH analysis.

The combination of universal PCR and CGH protocols possibly will enable us to study even single cells, such as Sternberg-Reed cells in Hodgkin's lymphoma. Moreover, upon physical isolation of cells from the same sample—differing, for example, in morphology or immunophenotype—it could become feasible to compare early and late stages of tumors within the same tissue, or even different clones within the same tumor.

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MULTIPLE-COLORED CHROMOSOMES BY FLUORESCENCE IN SITU HYBRIDIZATION

This section was contributed by J Wiegant and JG Dauwerse.

In situ hybridization makes it possible to detect specific nucleic acid sequences in morphologically preserved chromosomes, cells, and tissues. The technique is based on the duplex formation, under well-defined conditions, of a modified single-stranded nucleic acid fragment (probe) and its complementary sequence (target sequence) in a fixed biological specimen. The modification reaction of the probe renders the hybrid detectable via light microscopy.

The development of chemical and enzymatic modifications of nucleic acid probes with stable labels, which allow detection by fluorescence or an enzyme reaction, has resulted in the wide application of in situ hybridization (Lichter et al., 1991; Raap et al., 1990a, 1990b). These probes permit full use of the spatial resolution of the light microscope and its ability to visualize colors. Particularly in cytogenetics, fluorescence in situ hybridization (FISH) (Cornelisse et al., 1992; Emanuel, 1993; Raap et al., 1990c) is the method of choice because of its sensitivity and the possibility it affords to perform multiple hybridizations, because of the ability of fluorescence microscopy to visualize selectively different fluorochromes with spectrally well-separated emissions.

Two in situ hybridization methods are distinguished: the direct and the indirect (Wiegant et al., 1991, 1993). For both, chemical and enzymatic DNA modification reactions have been described (Lichter et al., 1991; Raap et al., 1990a). In general, an enzymatic approach is used for small-scale (100 ng to 5 μg) DNA modifications, whereas chemical procedures are recommended for large-scale synthesis.

In direct methods, the reporter molecule is bound to the nucleic acid probe so that the formed hybrids can be visualized microscopically immediately after the in situ hybridization.

For indirect methods, the probe is modified with an element (hapten), which enables the hybrids to be detected by affinity cytochemistry, and hence the characterization indirect. A number of such hapten modification reactions have been described, and if antibodies against the reporter molecules are available, direct methods also are amenable to immunocytochemical amplification.

Nucleic Acid Probes for FISH

A wide variety of nucleic acid probes are currently available for chromosome analysis by FISH, ranging from small (20 base pairs [bp]) synthetic oligo nucleotides to large (up to 1200 kilobase pairs [kbp]) genomic fragments cloned in yeast artificial chromosomes (YACs). Because the presence or absence of interspersed repeats in the probes determines whether or not a preannealing step has to be included in the FISH procedure (Landegent et al., 1987; Lichter et al., 1990b; Pinkel et al., 1988), and the chance of repeats being present in the probe increases as the complexity of the probe increases, from the FISH methodology point of view, we categorize the probes as high- or low-complexity probes. The complex probes are referred to as phage (15-kbp inserts), cosmid (40-kbp inserts), and YACs (up to 1.2 mbp) clones, as well as chromosome (fragment)–specific plasmid DNA libraries.

As stated, such high-complexity probes contain interspersed repetitive sequences such as the *Alu* and *Kpn* repeats that occur throughout the genome. For specific visualization of the unique elements in these probes, these repeats have to be eliminated from participation in the in situ hybridization. This is done in a so-called preannealing step. In solution, unlabeled competitor